PATENT APPLICATION
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POLYPEPTIDES AND ANTIBODIES DERIVED FROM CHRONIC LYMPHOCYTIC LEUKEMIA CELLS AND USES THEREOF

RELATED APPLICATIONS

This application is a continuation in part of U.S. Application Serial No. 10/379,151 filed on March 4, 2003 which, in turn, is a continuation in part of PCT/US01/47931 filed on December 10, 2001, which is an international application that claims priority to U. S. Provisional Application No. 60/254,113 filed December 8, 2000. The entire disclosures of the aforementioned U.S., international and provisional applications are incorporated herein by reference.

TECHNICAL FIELD

Cell lines derived from chronic lymphocytic leukemia (CLL) cells and the uses thereof in the study and treatment of CLL and other diseases are disclosed. In particular, this disclosure relates to a CLL cell line designated "CLL-AAT", deposited on November 28, 2001 with the American Type Culture Collection (Manassas, Virginia, USA) in accordance with the terms of the Budapest Treaty under ATCC accession no. PTA-3920.

BACKGROUND

Chronic Lymphocytic Leukemia (CLL) is a disease of the white blood cells and is the most common form of leukemia in the Western Hemisphere. CLL represents a diverse group of diseases relating to the growth of malignant lymphocytes that grow slowly but have an extended life span. CLL is classified in various categories that include, for example, B-cell chronic lymphocytic leukemia (B-CLL) of classical and mixed types, B-cell and T -cell prolymphocytic leukemia, hairy cell leukemia, and large granular lymphocytic leukemia.

Of all the different types of CLL, B-CLL accounts for approximately 30 percent of all leukemias. Although it occurs more frequently in individuals over 50 years of age, it is increasingly seen in younger people. B-CLL is characterized by accumulation of B-

lymphocytes that are morphologically normal but biologically immature, leading to a loss of function. Lymphocytes normally function to fight infection. In B-CLL, however, lymphocytes accumulate in the blood and bone marrow and cause swelling of the lymph nodes. The production of normal bone marrow and blood cells is reduced and patients often experience severe anemia as well as low platelet counts. This can pose the risk of life-threatening bleeding and the development of serious infections because of reduced numbers of white blood cells.

To further understand diseases such as leukemia it is important to have suitable cell lines that can be used as tools for research on their etiology, pathogenesis and biology. Examples of malignant human B-lymphoid cell lines include pre-B acute lymphoblasticleukemia (Reh), diffuse large cell lymphoma (WSU-DLCL2), and Waldenstrom's macroglobulinemia (WSU- WM). Unfortunately, many of the existing cell lines do not represent the clinically most common types of leukemia and lymphoma.

The use of Epstein Barr Virus (EBV) infection in vitro has resulted in some CLL derived cell lines, in particular B-CLL cells lines, that are representative of the malignant cells. The phenotype of these cell lines is different than that of the in vivo tumors and instead the features of B-CLL lines tend to be similar to those of Lymphoblastoid cell lines. Attempts to immortalize B-CLL cells with the aid of EBV infection have had little success. The reasons for this are unclear but it is known that it is not due to a lack of EBV receptor expression, binding or uptake. Wells et al. found that B-CLL cells were arrested in the G1/S phase of the cell cycle and that transformation associated EBV DNA was not expressed. This suggests that the interaction of EBV with B-CLL cells is different from that with normal B cells. EBV-transformed CLL cell lines moreover appear to differentiate, possessing a morphology more similar to lymphoblastoid cell lines (LCL) immortalized by EBV.

An EBV-negative CLL cell line, WSU-CLL, has been established previously (Mohammad et al., (1996) Leukemia 10(1):130-7). However, no other such cell lines are known.

Various mechanisms play a role in the body's response to a disease state, including cancer and CLL. For example, $CD4^{+}$T helper cells play a crucial role in an

effective immune response against various malignancies by providing stimulatory factors to effector cells. Cytotoxic T cells are believed to be the most effective cells to eliminate cancer cells, and T helper cells prime cytotoxic T cells by secreting Th1 cytokines such as IL-2 and IFN-γ. In various malignancies, T helper cells have been shown to have an altered phenotype compared to cells found in healthy individuals. One of the prominent altered features is decreased Th1 cytokine production and a shift to the production of Th2 cytokines. (See, e.g., Kiani, et al., *Haematologica* 88:754-761 (2003); Maggio, et al., *Ann Oncol* 13 Suppl 1:52-56 (2002); Ito, et al., *Cancer* 85:2359-2367 (1999); Podhorecka, et al., *Leuk Res* 26:657-660 (2002); Tatsumi, et al., *J Exp Med* 196:619-628 (2002); Agarwal, et al., *Immunol Invest* 32:17-30 (2003); Smyth, et al., *Ann Surg Oncol* 10:455-462 (2003); Contasta, et al., *Cancer Biother Radiopharm* 18:549-557 (2003); Lauerova, et al., *Neoplasma* 49:159-166(2002).) Reversing that cytokine shift to a Th1 profile has been demonstrated to augment anti-tumor effects of T cells. (See Winter, et al., *Immunology* 108:409-419 (2003); Inagawa, et al., *Anticancer Res* 18:3957-3964 (1998).)

Mechanisms underlying the capacity of tumor cells to drive the cytokine expression of T helper cells from Th1 to Th2 include the secretion of cytokines such as Il-10 or TGF-β as well as the expression of surface molecules interacting with cells of the immune system. OX-2/CD200, a molecule expressed on the surface of dendritic cells which possesses a high degree of homology to molecules of the immunoglobulin gene family, has been implicated in immune suppression (Gorczynski et al., *Transplantation* 65:1106-1114 (1998)) and evidence that OX-2/CD200-expressing cells can inhibit the stimulation of Th1 cytokine production has been provided. Gorczinski et al demonstrated in a mouse model that infusion of OX-2/CD200 Fc suppresses the rejection of tumor cells in an animal model using leukaemic tumor cells (*Clin Exp Immunol* 126:220-229 (2001)).

Improved methods for treating individuals suffering from cancer or CLL are desirable, especially to the extent they can enhance the activity of T cells.

SUMMARY

In one embodiment an CLL cell line of malignant origin is provided that is not established by immortalisation with EBV. The cell line, which was derived from primary CLL cells, and is deposited under ATCC accession no. PTA-3920. In a preferred embodiment, the cell line is CLL-AAT. CLL-AAT is B-CLL cell line, derived from a B-CLL primary cell.

In a further aspect, the CLL-AAT cell line is used to generate monoclonal antibodies useful in the diagnosis and/or treatment of CLL. Antibodies may be generated by using the cells as disclosed herein as immunogens, thus raising an immune response in animals from which monoclonal antibodies may be isolated. The sequence of such antibodies may be determined and the antibodies or variants thereof produced by recombinant techniques. In this aspect, "variants" includes chimeric, CDR-grafted, humanized and fully human antibodies based on the sequence of the monoclonal antibodies.

Moreover, antibodies derived from recombinant libraries ("phage antibodies") may be selected using the cells described herein, or polypeptides derived therefrom, as bait to isolate the antibodies on the basis of target specificity.

In a still further aspect, antibodies may be generated by panning antibody libraries using primary CLL cells, or antigens derived therefrom, and further screened and/or characterized using a CLL cell line, such as, for example, the CLL cell line described herein. Accordingly, a method for characterizing an antibody specific for CLL is provided, which includes assessing the binding of the antibody to a CLL cell line.

In a further aspect, there is provided a method for identifying proteins uniquely expressed in CLL cells employing the CLL-AAT cell line, by methods well known to those, skilled with art, such as by immunoprecipitation followed by mass spectroscopy analyses. Such proteins may be uniquely expressed in the CLL-AAT cell line, or in primary cells derived from CLL patients.

Small molecule libraries (many available commercially) may be screened using the CLL-AAT cell line in a cell-based assay to identify agents capable of modulating the growth characteristics of the cells. For example, the agents may be identified

which modulate apoptosis in the CLL-AAT cell line, or which inhibit growth and/or proliferation thereof. Such agents are candidates for the development of therapeutic compounds.

Nucleic acids isolated from CLL-AAT cell lines may be used in subtractive hybridization experiments to identify CLL-specific genes or in micro array analyses (e.g., gene chip experiments). Genes whose transcription is modulated in CLL cells may be identified. Polypeptide or nucleic acid gene products identified in this manner are useful as leads for the development of antibody or small molecule therapies for CLL.

In a preferred aspect, the CLL-AAT cell line may be used to identify internalizing antibodies, which bind to cell surface components which are internalized by the cell. Such antibodies are candidates for therapeutic use. In particular, single-chain antibodies, which remain stable in the cytoplasm and which retain intracellular binding activity, may be screened in this manner.

In yet another aspect, a therapeutic treatment is described in which a patient is screened for the presence of a polypeptide that is upregulated by a malignant cancer cell and an antibody that interferes with the metabolic pathway of the upregulated polypeptide is administered to the patient.

The present disclosure further is directed to methods wherein a determination is made as to whether OX-2/CD200 is upregulated in a subject and, if so, administering to the subject a polypeptide that binds to OX-2/CD200. In another embodiment, the polypeptide binds to an OX-2/CD200 receptor.

In another aspect, methods in accordance with this disclosure are used to treat a disease state in which OX-2/CD200 is upregulated in a subject by administering a polypeptide that binds to OX-2/CD200 or an OX-2/CD200 receptor to the subject afflicted with the disease state. In one embodiment, the disease state treated by these methods includes cancer, specifically, in other embodiments, CLL.

BRIEF DESCRIPTION OF THE FIGURES

Fig. 1. schematically illustrates typical steps involved in cell surface panning of antibody libraries by magnetically-activated cell sorting (MACS).

Fig. 2. is a graph showing the results of whole cell ELISA demonstrating binding of selected scFv clones to primary B-CLL cells and absence of binding to normal human PBMC. The designation 2°+3° in this and other figures refers to negative control wells stained with Mouse Anti-HA and detecting antimouse antibodies alone. The designation RSC-S Library in this and other figures refers to soluble antibodies prepared from original rabbit scFv unpanned library. The designation R3/RSC-S Pool in this and other figures refers to soluble antibodies prepared from entire pool of scFv antibodies from round 3 of panning. Anti-CD5 antibody was used as a positive control to verify that equal numbers of B-CLL and PBMC cells were plated in each well.

Figs. 3a and 3b show the results of whole cell ELISA comparing binding of selected scFv antibodies to primary B-CLL cells and normal primary human B cells. Anti-CD19 antibody was used as a positive control to verify that equal numbers of B-CLL and normal B cells were plated in each well. Other controls were as described in the legend to Fig 2.

Figs. 4a and 4b show the results of whole cell ELISA used to determine if scFv clones bind to patient-specific (i.e. idiotype) or blood type-specific (i.e. HLA) antigens. Each clone was tested for binding to PBMC isolated from 3 different B-CLL patients. Clones that bound to (1 patient sample were considered to be patient or blood type-specific.

Figs. 5a and 5b show the results of whole cell ELISA comparing binding of scFv clones to primary B-CLL cells and three human leukemic cell lines. Ramos is a mature B cell line derived from a Burkitt's lymphoma. RL is a mature B cell line derived from a non-Hodgkin's lymphoma. TF-I is an erythroblastoid cell line derived from a erythroleukemia.

Figs. 6a, 6b and 6c show the results of whole cell ELISA comparing binding of scFv clones to primary B-CLL cells and CLL-AAT, a cell line derived from a B-CLL patient. TF-I cells were included as a negative control.

Fig. 7 shows the binding specificity of scFv antibodies in accordance with this disclosure as analyzed by 3-color flow cytometry. In normal peripheral blood mononuclear cells, the antigen recognized by scFv-9 is moderately expressed on B lymphocytes and weakly expressed on a subpopulation of T lymphocytes. PBMC from

a normal donor were analyzed by 3-color flow cytometry using anti-CD5-FITC, anti-CD19-PerCP, and scFv-9/Anti-HA-biotin/streptavidin-PE.

Figs. 8a, 8b and 8c show the expression levels of antigens recognized by scFv antibodies in accordance with this disclosure. The antigens recognized by scFv-3 and scFv-9 are overexpressed on the primary CLL tumor from which the CLL-AAT cell line was derived. Primary PBMC from the CLL patient used to establish the CLL-AAT cell line or PBMC from a normal donor were stained with scFv antibody and analyzed by flow cytometry. ScFv-3 and scFv-9 stain the CLL cells more brightly than the normal PBMC as measured by the mean fluorescent intensities.

Figs. 9a and 9b provide a summary of CDR sequences and binding specificities of selected scFv antibodies.

Fig 10. is Table 2 which shows a summary of flow cytometry results comparing expression levels of scFv antigens on primary CLL cells vs. normal PBMC as described in Figs 8a-8c.

Fig 11. is a Table showing a summary of flow cytometry results comparing expression levels of scFv-9 antigen with the percentage of CD38+ cells in peripheral blood mononuclear cells isolated from ten CLL patients.

Fig 12. shows the identification of scFv antigens by immunoprecipitation and mass spectrometry. CLL-AAT cells were labeled with a solution of 0.5mg/ml sulfo-NHS-LC-biotin (Pierce) in PBS, pH8.0 for 30'. After extensive washing with PBS to remove unreacted biotin, the cells were disrupted by nitrogen cavitation and the microsomal fraction was isolated by differential centrifugation. The microsomal fraction was resuspended in NP40 Lysis Buffer and extensively precleared with normal rabbit serum and protein A sepharose. Antigens were immunoprecipitated with HA-tagged scFv antibodies coupled to Rat Anti-HA agarose beads (Roche). Following immunoprecipitation, antigens were separated by SDS-PAGE and detected by Western blot using streptavidin-alkaline phosphatase(AP) or by Coomassie G-250 staining. ScFv-7, an antibody which doesn't bind to CLL-AAT cells, was used as a negative control. Antigen bands were excised from the Coomassie-stained gel and identified by mass spectrometry (MS). MALDI-MS was performed at the Proteomics

Core Facility of The Scripps Research Institute (La Jolla, CA). μLC/MS/MS was performed at the Harvard Microchemistry Facility (Cambridge, MA).

Fig 13. shows that three scFv antibodies bind specifically to 293-EBNA cells transiently transfected with a human OX-2/CD200 cDNA clone. A OX-2/CD200 cDNA was cloned from CLL cells by RT-PCR and inserted into the mammalian expression vector pCEP4 (Invitrogen). PCEP4-CD200 plasmid or the corresponding empty vector pCEP4 was transfected into 293-EBNA cells using Polyfect reagent (QIAGEN). Two days after transfection, the cells were analyzed for binding to scFv antibodies by flow cytometry.

Fig 14. shows that the presence of OX-2/CD200 transfected cells resulted in down-regulation of Th1 cytokines such as IL-2 and IFN-γ. Addition of the anti-OX-2/CD200 antibody at 30 μg/ml fully restored the Th1 response.

Fig. 15 shows that the presence of CLL cells in a mixed lymphocyte reaction resulted in down-regulation of the Th1 response for IL-2.

Fig. 16 shows that the presence of CLL cells in a mixed lymphocyte reaction resulted in down-regulation of the Th1 response for IFN-γ.

DETAILED DESCRIPTION

In accordance with the present disclosure, methods are provided for determining whether OX-2/CD200 is upregulated in a subject and, if so, administering to the subject a polypeptide that binds to OX-2/CD200. In general, the polypeptides utilized in the present disclosure can be constructed using different techniques which are known to those skilled in the art. In one embodiment, the polypeptides are obtained by chemical synthesis. In other embodiments, the polypeptides are antibodies or constructed from a fragment or several fragments of one or more antibodies.

Preferably, the polypeptides utilized in the methods of the present disclosure are obtained from a CLL cell line. "CLL", as used herein, refers to chronic lymphocytic leukemia involving any lymphocyte including, but not limited to, various developmental stages of B cells and T cells including, but not limited to, B cell CLL ("B-CLL"). B-CLL, as used herein, refers to leukemia with a mature B cell phenotype which is $CD5^+$,

$CD23^+$, $CD20^{dim+}$, sIg^{dim+} and arrested in G0/G1 of the cell cycle. In a further aspect, the CLL cell line is used to generate polypeptides, including antibodies, useful in the diagnosis and/or treatment of a disease state in which OX-2/CD200 is upregulated, including cancer and CLL.

As used herein, the term "antibodies" refers to complete antibodies or antibody fragments capable of binding to a selected target. Included are Fv, scFv, Fab' and F(ab')2, monoclonal and polyclonal antibodies, engineered antibodies (including chimeric, CDR-grafted and humanized, fully human antibodies, and artificially selected antibodies), and synthetic or semi-synthetic antibodies produced using phage display or alternative techniques. Small fragments, such as Fv and scFv, possess advantageous properties for diagnostic and therapeutic applications on account of their small size and consequent superior tissue distribution.

The polypeptides and/or antibodies utilized herein are especially indicated for diagnostic and therapeutic applications. Accordingly, they may be altered with an effector protein such as a toxin or a label. Especially preferred are labels which allow the imaging of the distribution of the polypeptide or antibody in vivo. Such labels may be radioactive labels or radiopaque labels, such as metal particles, which are readily visualisable within the body of a patient. Moreover, the labels may be fluorescent labels or other labels which are visualisable on tissue samples removed from patients.

Antibodies may be generated by using the cells as disclosed herein as immunogens, thus raising an immune response in animals from which monoclonal antibodies may be isolated. The sequence of such antibodies may be determined and the antibodies or variants thereof produced by recombinant techniques. In this aspect, "variants" includes chimeric, CDR-grafted, humanized and fully human antibodies based on the sequence of the monoclonal antibodies, as well as polypeptides capable of binding to OX-2/CD200.

Moreover, antibodies derived from recombinant libraries ("phage antibodies") may be selected using the cells described herein, or polypeptides derived therefrom, as bait to isolate the antibodies or polypeptides on the basis of target specificity.

In a still further aspect, antibodies or polypeptides may be generated by panning antibody libraries using primary CLL cells, or antigens derived therefrom, and

further screened and/or characterized using a CLL cell line, such as, for example, the CLL cell line described herein. Accordingly, a method for characterizing an antibody or polypeptide specific for CLL is provided, which includes assessing the binding of the antibody or polypeptide to a CLL cell line.

Preparation of Cell Lines

Cell lines may be produced according to established methodologies known to those skilled in the art. In general, cell lines are produced by culturing primary cells derived from a patient until immortalized cells are spontaneously generated in culture. These cells are then isolated and further cultured to produce clonal cell populations or cells exhibiting resistance to apoptosis.

For example, CLL cells may be isolated from peripheral blood drawn from a patient suffering from CLL. The cells may be washed, and optionally immunotyped in order to determine the type(s) of cells present. Subsequently, the cells may be cultured in a medium, such as a medium containing IL-4. Advantageously, all or part of the medium is replaced one or more times during the culture process. Cell lines may be isolated thereby, and will be identified by increased growth in culture.

In one embodiment a CLL cell line of malignant origin is provided that is not established by immortalization with EBV. "Malignant origin" refers to the derivation of the cell line from malignant CLL primary cells, as opposed to non-proliferating cells which are transformed, for example, with EBV. Cell lines useful according to this disclosure may be themselves malignant in phenotype, or not. A CLL cell having a "malignant" phenotype encompasses cell growth unattached from substrate media characterized by repeated cycles of cell growth and exhibits resistance to apoptosis. The cell line, which was derived from primary CLL cells, is deposited under ATCC accession no. PTA-3920. In a preferred embodiment, the cell line is CLL-AAT. CLL-AAT is B-CLL cell line, derived from a B-CLL primary cell.

In one embodiment, proteins uniquely expressed in CLL cells are identified employing the CLL-AAT cell line by methods well known to those skilled in the art, such as by immunoprecipitation followed by mass spectroscopy analyses. Such

proteins may be uniquely expressed in the CLL-AAT cell line, or in primary cells derived from CLL patients.

Small molecule libraries (many available commercially) may be screened using the CLL-AAT cell line in a cell-based assay to identify agents capable of modulating the growth characteristics of the cells. For example, the agents may be identified which modulate apoptosis in the CLL-AAT cell line, or which inhibit growth and/or proliferation thereof. Such agents are candidates for the development of therapeutic compounds.

Nucleic acids isolated from CLL-AAT cell lines may be used in subtractive hybridization experiments to identify CLL-specific genes or in micro array analyses (e.g., gene chip experiments). Genes whose transcription is modulated in CLL cells may be identified. Polypeptide or nucleic acid gene products identified in this manner are useful as leads for the development of antibody or small molecule therapies for CLL.

In one embodiment, the CLL-AAT cell line may be used to identify internalizing antibodies, which bind to cell surface components and are then internalized by the cell. Such antibodies are candidates for therapeutic use. In particular, single-chain antibodies, which remain stable in the cytoplasm and which retain intracellular binding activity, may be screened in this manner.

Preparation of Monoclonal Antibodies

Recombinant DNA technology may be used to improve the antibodies produced in accordance with this disclosure. Thus, chimeric antibodies may be constructed in order to decrease the immunogenicity thereof in diagnostic or therapeutic applications. Moreover, immunogenicity may be minimized by humanizing the antibodies by CDR grafting and, optionally, framework modification. See, U.S. Patent No. 5,225,539, the contents of which are incorporated herein by reference.

Antibodies may be obtained from animal serum, or, in the case of monoclonal antibodies or fragments thereof produced in cell culture. Recombinant DNA technology may be used to produce the antibodies according to established procedure, in

bacterial or preferably mammalian cell culture. The selected cell culture system preferably secretes the antibody product.

In another embodiment, a process for the production of an antibody disclosed herein includes culturing a host, e.g. E. coli or a mammalian cell, which has been transformed with a hybrid vector. The vector includes one or more expression cassettes containing a promoter operably linked to a first DNA sequence encoding a signal peptide linked in the proper reading frame to a second DNA sequence encoding the antibody protein. The antibody protein is then collected and isolated. Optionally, the expression cassette may include a promoter operably linked to polycistronic, for example bicistronic, DNA sequences encoding antibody proteins each individually operably linked to a signal peptide in the proper reading frame.

Multiplication of hybridoma cells or mammalian host cells in vitro is carried out in suitable culture media, which include the customary standard culture media (such as, for example Dulbecco's Modified Eagle Medium (DMEM) or RPMI 1640 medium), optionally replenished by a mammalian serum (e.g. fetal calf serum), or trace elements and growth sustaining supplements (e.g. feeder cells such as normal mouse peritoneal exudate cells, spleen cells, bone marrow macrophages, 2-aminoethanol, insulin, transferrin, low density lipoprotein, oleic acid, or the like). Multiplication of host cells which are bacterial cells or yeast cells is likewise carried out in suitable culture media known in the art. For example, for bacteria suitable culture media include medium LE, NZCYM, NZYM, NZM, Terrific Broth, SOB, SOC, 2 x YT, or M9 Minimal Medium. For yeast, suitable culture media include medium YPD, YEPD, Minimal Medium, or Complete Minimal Dropout Medium.

In vitro production provides relatively pure antibody preparations and allows scale-up to give large amounts of the desired antibodies. Techniques for bacterial cell, yeast, plant, or mammalian cell cultivation are known in the art and include homogeneous suspension culture (e.g. in an airlift reactor or in a continuous stirrer reactor), and immobilized or entrapped cell culture (e.g. in hollow fibres, microcapsules, on agarose microbeads or ceramic cartridges).

Large quantities of the desired antibodies can also be obtained by multiplying mammalian cells in vivo. For this purpose, hybridoma cells producing the desired

antibodies are injected into histocompatible mammals to cause growth of antibody-producing tumors. Optionally, the animals are primed with a hydrocarbon, especially mineral oils such as pristane (tetramethyl-pentadecane), prior to the injection. After one to three weeks, the antibodies are isolated from the body fluids of those mammals. For example, hybridoma cells obtained by fusion of suitable myeloma cells with antibody-producing spleen cells from Balb/c mice, or transfected cells derived from hybridoma cell line Sp2/0 that produce the desired antibodies are injected intraperitoneally into Balb/c mice optionally pre-treated with pristine. After one to two weeks, ascitic fluid is taken from the animals.

The foregoing, and other, techniques are discussed in, for example, Kohler and Milstein, (1975) Nature 256:495-497; U.S. Patent No. 4,376,110; Harlow and Lane, Antibodies: a Laboratory Manual, (1988) Cold Spring Harbor, the disclosures of which are all incorporated herein by reference. Techniques for the preparation of recombinant antibody molecules is described in the above references and also in, for example WO97/08320; U.S. Patent No. 5,427,908; U.S. Patent No. 5,508,717; Smith, 1985, Science, Vol. 225, pp 1315-1317; Parmley and Smith 1988, Gene 73, pp 305-318; De La Cruz et al, 1988, Journal of Biological Chemistry, 263 pp 4318-4322; U.S. Patent No. 5,403,484; U.S. Patent No. 5223409; WO88/06630; WO92/15679; U.S. Patent No. 5780279; U.S. Patent No. 5571698; U.S. Patent No. 6040136; Davis et al., Cancer Metastasis Rev.,1999;18(4):421-5; Taylor, et al., Nucleic Acids Research 20 (1992): 6287-6295; Tomizuka et al.,Proc. Nat. Academy of Sciences USA 97(2) (2000): 722-727. The contents of all these references are incorporated herein by reference.

The cell culture supernatants are screened for the desired antibodies, preferentially by immunofluorescent staining of CLL cells, by immunoblotting, by an enzyme immunoassay, e.g. a sandwich assay or a dot-assay, or a radioimmunoassay.

For isolation of the antibodies, the immunoglobulins in the culture supernatants or in the ascitic fluid may be concentrated, e.g. by precipitation with ammonium sulfate, dialysis against hygroscopic material such as polyethylene glycol, filtration through selective membranes, or the like. If necessary and/or desired, the antibodies are purified by the customary chromatography methods, for example gel filtration, ion-

exchange chromatography, chromatography over DEAE-cellulose and/or (immuno-) affinity chromatography, e.g. affinity chromatography with a one or more surface polypeptides derived from a CLL cell line according to this disclosure, or with Protein-A or G.

Another embodiment provides a process for the preparation of a bacterial cell line secreting antibodies directed against the cell line characterized in that a suitable mammal, for example a rabbit, is immunized with pooled CLL patient samples. A phage display library produced from the immunized rabbit is constructed and panned for the desired antibodies in accordance with methods well known in the art (such as, for example, the methods disclosed in the various references incorporated herein by reference).

Hybridoma cells secreting the monoclonal antibodies are also contemplated. The preferred hybridoma cells are genetically stable, secrete monoclonal antibodies described herein of the desired specificity and can be activated from deep-frozen cultures by thawing and recloning.

In another embodiment, a process is provided for the preparation of a hybridoma cell line secreting monoclonal antibodies directed to the CLL cell line is described herein. In that process, a suitable mammal, for example a Balb/c mouse, is immunized with a one or more polypeptides or antigenic fragments thereof derived from a cell described in this disclosure, the cell line itself, or an antigenic carrier containing a purified polypeptide as described. Antibody-producing cells of the immunized mammal are grown briefly in culture or fused with cells of a suitable myeloma cell line. The hybrid cells obtained in the fusion are cloned, and cell clones secreting the desired antibodies are selected. For example, spleen cells of Balb/c mice immunized with the present cell line are fused with cells of the myeloma cell line PAI or the myeloma cell line Sp2/0-Ag 14, the obtained hybrid cells are screened for secretion of the desired antibodies, and positive hybridoma cells are cloned.

Preferred is a process for the preparation of a hybridoma cell line, characterized in that Balb/c mice are immunized by injecting subcutaneously and/or intraperitoneally between 10^6 and 10^7 cells of a cell line in accordance with this disclosure several times, e.g. four to six times, over several months, e.g. between two and four months.

Spleen cells from the immunized mice are taken two to four days after the last injection and fused with cells of the myeloma cell line PAI in the presence of a fusion promoter, preferably polyethylene glycol. Preferably, the myeloma cells are fused with a three- to twenty-fold excess of spleen cells from the immunized mice in a solution containing about 30% to about 50% polyethylene glycol of a molecular weight around 4000. After the fusion, the cells are expanded in suitable culture media as described hereinbefore, supplemented with a selection medium, for example HAT medium, at regular intervals in order to prevent normal myeloma cells from overgrowing the desired hybridoma cells.

In a further embodiment, recombinant DNA comprising an insert coding for a heavy chain variable domain and/or for a light chain variable domain of antibodies directed to the cell line described hereinbefore are produced. The term DNA includes coding single stranded DNAs, double stranded DNAs consisting of said coding DNAs and of complementary DNAs thereto, or these complementary (single stranded) DNAs themselves.

Furthermore, DNA encoding a heavy chain variable domain and/or a light chain variable domain of antibodies directed to the cell line disclosed herein can be enzymatically or chemically synthesized DNA having the authentic DNA sequence coding for a heavy chain variable domain and/or for the light chain variable domain, or a mutant thereof. A mutant of the authentic DNA is a DNA encoding a heavy chain variable domain and/or a light chain variable domain of the above-mentioned antibodies in which one or more amino acids are deleted or exchanged with one or more other amino acids. Preferably said modification(s) are outside the CDRs of the heavy chain variable domain and/or of the light chain variable domain of the antibody in humanization and expression optimization applications. The term mutant DNA also embraces silent mutants wherein one or more nucleotides are replaced by other nucleotides with the new codons coding for the same amino acid(s). The term mutant sequence also includes a degenerated sequence. Degenerated sequences are degenerated within the meaning of the genetic code in that an unlimited number of nucleotides are replaced by other nucleotides without resulting in a change of the amino acid sequence originally encoded. Such degenerated sequences may be useful

due to their different restriction sites and/or frequency of particular codons which are preferred by the specific host, particularly E. coli, to obtain an optimal expression of the heavy chain murine variable domain and/or a light chain murine variable domain.

The term mutant is intended to include a DNA mutant obtained by in vitro mutagenesis of the authentic DNA according to methods known in the art.

For the assembly of complete tetrameric immunoglobulin molecules and the expression of chimeric antibodies, the recombinant DNA inserts coding for heavy and light chain variable domains are fused with the corresponding DNAs coding for heavy and light chain constant domains, then transferred into appropriate host cells, for example after incorporation into hybrid vectors.

Recombinant DNAs including an insert coding for a heavy chain murine variable domain of an antibody directed to the cell line disclosed herein fused to a human constant domain g, for example $\gamma 1$, $\gamma 2$, $\gamma 3$ or $\gamma 4$, preferably $\gamma 1$ or $\gamma 4$ are also provided. Recombinant DNAs including an insert coding for a light chain murine variable domain of an antibody directed to the cell line disclosed herein fused to a human constant domain κ or λ, preferably κ are also provided

Another embodiment pertains to recombinant DNAs coding for a recombinant polypeptide wherein the heavy chain variable domain and the light chain variable domain are linked by way of a spacer group, optionally comprising a signal sequence facilitating the processing of the antibody in the host cell and/or a DNA coding for a peptide facilitating the purification of the antibody and/or a cleavage site and/or a peptide spacer and/or an effector molecule.

The DNA coding for an effector molecule is intended to be a DNA coding for the effector molecules useful in diagnostic or therapeutic applications. Thus, effector molecules which are toxins or enzymes, especially enzymes capable of catalyzing the activation of prodrugs, are particularly indicated. The DNA encoding such an effector molecule has the sequence of a naturally occurring enzyme or toxin encoding DNA, or a mutant thereof, and can be prepared by methods well known in the art.

Antibodies and antibody fragments disclosed herein are useful in diagnosis and therapy. Accordingly, a composition for therapy or diagnosis comprising an antibody disclosed herein is provided.

In the case of a diagnostic composition, the antibody is preferably provided together with means for detecting the antibody, which may be enzymatic, fluorescent, radioisotopic or other means. The antibody and the detection means may be provided for simultaneous, separate or sequential use, in a diagnostic kit intended for diagnosis.

While the above disclosure has been directed to antibodies, in some embodiments polypeptides derived from such antibodies can be utilized in accordance with the present disclosure.

Uses of the CLL Cell Line

There are many advantages to the development of a CLL cell line, as it provides an important tool for the development of diagnostics and treatments for CLL, cancer, and other disease states characterized by upregulated levels of OX-2/CD200, e.g., melanoma.

A cell line according to this disclosure may be used for in vitro studies on the etiology, pathogenesis and biology of CLL and other disease states characterized by upregulated levels of OX-2/CD200. This assists in the identification of suitable agents that are useful in the therapy of these diseases.

The cell line may also be used to produce polypeptides and/or monoclonal antibodies for in vitro and in vivo diagnosis of CLL, cancer, and other disease states characterized by upregulated levels of OX-2/CD200 (e.g., melanoma), as referred to above, and for the screening and/or characterization of antibodies produced by other methods, such as by panning antibody libraries with primary cells and/or antigens derived from CLL patients.

The cell line may be used as such, or antigens may be derived therefrom. Advantageously, such antigens are cell-surface antigens specific for CLL. They may be isolated directly from cell lines according to this disclosure. Alternatively, a cDNA expression library made from a cell line described herein may be used to express CLL-specific antigens, useful for the selection and characterization of anti-CLL antibodies and the identification of novel CLL-specific antigens.

Treatment of CLL using monoclonal antibody therapy has been proposed in the art. Recently, Hainsworth (Oncologist 5 (5) (2000) 376-384) has described the current

therapies derived from monoclonal antibodies. Lymphocytic leukemia in particular is considered to be a good candidate for this therapeutic approach due to the presence of multiple lymphocyte-specific antigens on lymphocyte tumors.

Existing antibody therapies (such as RituximabTM, directed against the CD20-antigen, which is expressed on the surface of B-lymphocytes) have been used successfully against certain lymphocytic disease. However, a lower density CD20 antigen is expressed on the surface of B-lymphocytes in CLL (Almasri et al., Am. J. Hematol., 40 (4) (1992) 259-263).

The CLL cell line described herein thus permits the development of novel anti-CLL antibodies and polypeptides having specificity for one or more antigenic determinants of the present CLL cell line, and their use in the therapy and diagnosis of CLL, cancer, and other disease states characterized by upregulated levels of OX-2/CD200.

The antibody or polypeptide may bind to a receptor with which OX-2/CD200 normally interacts, thereby preventing or inhibiting OX-2/CD200 from binding to the receptor. As yet another alternative, the antibody can bind to an antigen that modulates expression of OX-2/CD200, thereby preventing or inhibiting normal or increased expression of OX-2/CD200. Because the presence of OX-2/CD200 has been associated with reduced immune response, it would be desirable to interfere with the metabolic pathway of OX-2/CD200 so that the patient's immune system can defend against the disease state, such as cancer or CLL, more effectively.

In a particularly useful embodiment, the polypeptide binds to OX-2/CD200. In one embodiment, the polypeptide can be an antibody which binds to OX-2/CD200 and prevents or inhibits OX-2/CD200 from interacting with other molecules or receptors. As CLL cells and other cells overexpressing OX-2/CD200 greatly diminish the production of Th1 cytokines, the administration of anti-CD200 antibody or a polypeptide which binds to OX-2/CD200 to a subject having upregulated levels of OX-2/CD200 restores the Th1 cytokine profile. Thus, these polypeptides and/or antibodies can be useful therapeutic agents in the treatment of CLL and other cancers or diseases over-expressing OX-2/CD200.

Thus, in another embodiment, the method of the present disclosure includes the steps of screening a subject for the presence OX-2/CD200 and administering a polypeptide that binds to OX-2/CD200. In a particularly useful embodiment, a CLL patient is screened for overexpression of OX-2/CD200 and an antibody that binds to OX-2/CD200 is administered to the patient. As described in detail below, one such antibody is scFv-9 (see Fig. 9B) which binds to OX-2/CD200.

In order that those skilled in the art may be better able to practice the compositions and methods described herein, the following examples are given for illustration purposes.

EXAMPLE 1

Isolation of Cell Line CLL-AAT

Establishment of the cell line

Peripheral blood from a patient diagnosed with CLL was obtained. The WBC count was $1.6x10^8$/ml. Mononuclear cells were isolated by Histopaque-1077 density gradient centrifugation (Sigma Diagnostics, St. Louis, MO). Cells were washed twice with Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), and resuspended in 5 ml of ice-cold IMDM/10% FBS. Viable cells were counted by staining with trypan blue. Cells were mixed with an equal volume of 85% FBS/15% DMSO and frozen in 1 ml aliquots for storage in liquid nitrogen.

Immunophenotyping showed that >90% of the CD45+ lymphocyte population expressed IgD, kappa light chain, CD5, CD19, and CD23. This population also expressed low levels of IgM and CD20. Approximately 50% of the cells expressed high levels of CD38. The cells were negative for lambda light chain, CD10 and CD138

An aliquot of the cells was thawed, washed, and resuspended at a density of 10^7/mL in IMDM supplemented with 20% heat-inactivated FBS, 2mM L-glutamine, 100 units/ml penicillin, 100 µg/ml streptomycin, 50 µM 2-mercaptoethanol, and 5 ng/ml recombinant human IL-4 (R & D Systems, Minneapolis, MN). The cells were cultured at 37°C in a humidified 5% CO2 atmosphere. The medium was partially replaced every 4 days until steady growth was observed. After 5 weeks, the number of cells in

the culture began to double approximately every 4 days. This cell line was designated CLL-AAT.

Characterization of the cell line

Immunophenotyping of the cell line by flow cytometry showed high expression of IgM, kappa light chain, CD23, CD38, and CD138, moderate expression of CD19 and CD20, and weak expression of IgD and CD5. The cell line was negative for lambda light chain, CD4, CD8, and CD10.

Immunophenotyping of the cell line was also done by whole cell ELISA using a panel of rabbit scFv antibodies that had been selected for specific binding to primary B-CLL cells. All of these CLL-specific scFv antibodies also recognized the CLL -AAT cell line. In contrast, the majority of the scFvs did not bind to two cell lines derived from B cell lymphomas: Ramos, a Burkitt's lymphoma cell line, and RL, a non-Hodgkin's lymphoma cell line.

EXAMPLE 2

Selection of scFv Antibodies for B-CLL-specific Cell Surface Antigens using Antibody Phage Display and Cell Surface Panning

Immunizations and scFv antibody library construction

Peripheral blood mononuclear cells (PBMC) were isolated from blood drawn from CLL patients at the Scripps Clinic (La Jolla, CA). Two rabbits were immunized with $2x10^7$ PBMC pooled from 10 different donors with CLL. Three immunizations, two sub-cutaneous injections followed by one intravenous injection, were done at three week intervals. Serum titers were checked by measuring binding of serum IgG to primary CLL cells using flow cytometry. Five days after the final immunization, spleen, bone marrow, and PBMC were harvested from the animals. Total RNA was isolated from these tissues using Tri-Reagent (Molecular Research Center, Inc). Single-chain Fv (scFv) antibody phage display libraries were constructed as previously described (Barbas et al., (2001) Phage Display: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York). For cell surface panning, phagemid particles from the reamplified library were precipitated with polyethylene

glycol (PEG), resuspended in phosphate-buffered saline(PBS) containing 1% bovine serum albumin (BSA), and dialysed overnight against PBS.

Antibody selection by cell surface panning

The libraries were enriched for CLL cell surface-specific antibodies by positive-negative selection with a magnetically-activated cell sorter (MACS) as described by Siegel et al.(1997, J. Immunol. Methods 206:73-85). Briefly, phagemid particles from the scFv antibody library were preincubated in MPBS (2% nonfat dry milk, 0.02% sodium azide in PBS, pH 7.4) for 1 hour at 25°C to block nonspecific binding sites. Approximately 10^7 primary CLL cells were labeled with mouse anti-CD5 IgG and mouse anti-CD19 IgG conjugated to paramagnetic microbeads (Miltenyi Biotec, Sunnyvale, CA). Unbound microbeads were removed by washing. The labeled CLL cells ("target cells") were mixed with an excess of "antigen-negative absorber cells", pelleted, and resuspended in 50µl (10^{10}-10^{11} cfu) of phage particles. The absorber cells serve to soak up phage that stick non-specifically to cell surfaces as well as phage specific for "common" antigens present on both the target and absorber cells. The absorber cells used were either TF-1 cells (a human erythroleukemia cell line) or normal human B cells isolated from peripheral blood by immunomagnetic negative selection (StemSep system, StemCell Technologies, Vancouver, Canada). The ratio of absorber cells to target cells was approximately 10 fold by volume. After a 30 minute incubation at 25°C, the cell/phage mixture was transferred to a MiniMACS MS^+ separation column. The column was washed twice with 0.5 ml of MPBS, and once with 0.5 ml of PBS to remove the unbound phage and absorber cells. The target cells were eluted from the column in 1 ml of PBS and pelleted in a microcentrifuge at maximum speed for 15 seconds. The captured phage particles were eluted by resuspending the target cells in 200 µl of acid elution buffer (0.1 N HCl, pH adjusted to 2.2 with glycine, plus1 µg/ml BSA). After a 10 minute incubation at 25°C, the buffer was neutralized with 12 µL of 2M Tris base, pH10.5, and the eluted phage were amplified in E. coli for the next round of panning. For each round of panning, the input and output phage titers were determined. The input titer is the number of reamplified phage particles added to the target cell/absorber cell mixture and the output titer is the

number of captured phage eluted from the target cells. An enrichment factor (E) is calculated using the formula $E=(R_n \text{ output}/R_n \text{ input})/(R_1 \text{ output}/R_1 \text{ input})$. In most cases, an enrichment factor of 10^2-10^3 fold should be attained by the third or fourth round.

Analysis of enriched antibody pools following panning

After 3-5 rounds of panning, the pools of captured phage were assayed for binding to CLL cells by flow cytometry and/or whole cell ELISA:

1. To produce an entire pool in the form of HA-tagged soluble antibodies, 2ml of a non-suppressor strain of E. coli (e.g. TOP10F') was infected with 1µl (10^9-10^{10} cfu) of phagemid particles. The original, unpanned library was used as a negative control. Carbenicillin was added to a final concentration of 10µM and the culture was incubated at 37°C with shaking at 250rpm for 1 hour. Eight ml of SB medium containing 50µg/ml carbenicillin was added and the culture was grown to an OD 600 of ~0.8. IPTG was added to a final concentration of 1mM to induce scFv expression from the Lac promoter and shaking at 37°C was continued for 4 hours. The culture was centrifuged at 3000xg for 15'. The supernatant containing the soluble antibodies was filtered and stored in 1 ml aliquots at –20°C.
2. Binding of the scFv antibody pools to target cells vs. absorber cells was determined by flow cytometry using high-affinity Rat Anti-HA (clone 3F10, Roche Molecular Biochemicals) as secondary antibody and PE-conjugated Donkey Anti-Rat as tertiary antibody.
3. Binding of the antibody pools to target cells vs. absorber cells was also determined by whole-cell ELISA as described below.

Screening individual scFv clones following panning

To screen individual scFv clones following panning, TOP10F' cells were infected with phage pools as described above, spread onto LB plates containing carbenicillin and tetracycline, and incubated overnight at 37°C. Individual colonies were inoculated into deep 96-well plates containing 0.6-1.0 ml of SB-carbenicillin medium per well. The cultures were grown for 6-8 hours in a HiGro shaking incubator

(GeneMachines, San Carlos, CA) at 520 rpm and 37°C. At this point, a 90 µl aliquot from each well was transferred to a deep 96-well plate containing 10 µL of DMSO. This replica plate was stored at –80°C. IPTG was added to the original plate to a final concentration of 1 mM and shaking was continued for 3 hours. The plates were centrifuged at 3000xg for 15 minutes. The supernatants containing soluble scFv antibodies were transferred to another deep 96-well plate and stored at –20°C.

A sensitive whole-cell ELISA method for screening HA-tagged scFv antibodies was developed:

1. An ELISA plate is coated with concanavalin A (10mg/ml in 0.1 M $NaHCO_3$, pH8.6, 0.1 mM $CaCl_2$).
2. After washing the plate with PBS, 0.5-1x10^5 target cells or absorber cells in 50µl of PBS are added to each well, and the plate is centrifuged at 250xg for 10 minutes.
3. 50µl of 0.02% glutaraldehyde in PBS are added and the cells are fixed overnight at 4°C.
4. After washing with PBS, non-specific binding sites are blocked with PBS containing 4% non-fat dry milk for 3 hours at room temperature.
5. The cells are incubated with 50µl of soluble, HA-tagged scFv or Fab antibody (TOP10F' supernatant) for 2 hours at room temperature, then washed six times with PBS.
6. Bound antibodies are detected using a Mouse Anti-HA secondary antibody (clone 12CA5) and an alkaline phosphatase (AP)-conjugated Anti-Mouse IgG tertiary antibody. An about 10-fold amplification of the signal is obtained by using AMDEX AP-conjugated Sheep Anti-Mouse IgG as the tertiary antibody (Amersham Pharmacia Biotech). The AMDEX antibody is conjugated to multiple AP molecules via a dextran backbone. Color is developed with the alkaline phosphatase substrate PNPP and measured at 405nm using a microplate reader.

Primary screening of the scFv clones was done by ELISA on primary CLL cells versus normal human PBMC. Clones which were positive on CLL cells and negative on normal PBMC were rescreened by ELISA on normal human B cells, human B cell lines, TF-1 cells, and the CLL-AAT cell line. The clones were also rescreened by ELISA on CLL cells isolated from three different patients to eliminate clones that

recognized patient-specific or blood type-specific antigens. Results from representative ELISAs are shown in Figures 2-6 and summarized in Figure 9.

The number of unique scFv antibody clones obtained was determined by DNA fingerprinting and sequencing. The scFv DNA inserts were amplified from the plasmids by PCR and digested with the restriction enzyme BstNI. The resulting fragments were separated on a 4% agarose gel and stained with ethidium bromide. Clones with different restriction fragment patterns must have different amino acid sequences. Clones with identical patterns probably have similar or identical sequences. Clones with unique BstNI fingerprints were further analyzed by DNA sequencing. Twenty-five different sequences were found, which could be clustered into 16 groups of antibodies with closely related complementarity determining regions (Figure 9).

Characterization of scFv antibodies by flow cytometry

The binding specificities of several scFv antibodies were analyzed by 3-color flow cytometry (Fig. 7). PBMC isolated from normal donors were stained with FITC-conjugated anti-CD5 and PerCP-conjugated anti-CD19. Staining with scFv antibody was done using biotin-conjugated anti-HA as secondary antibody and PE-conjugated streptavidin. Three antibodies, scFv-2, scFv-3, and scFv-6, were found to specifically recognize the $CD19^+$ B lymphocyte population (data not shown). The fourth antibody, scFv-9, recognized two distinct cell populations: the $CD19^+$ B lymphocytes and a subset of $CD5^+$ T lymphocytes (Fig 7). Further characterization of the T cell subset showed that it was a subpopulation of the $CD4^+CD8^-$ T_H cells (data not shown).

To determine if the antigens recognized by the scFv antibodies were overexpressed on primary CLL cells, PBMC from five CLL patients and five normal donors were stained with scFv and compared by flow cytometry (Fig 8 and Table 2). By comparing the mean fluorescent intensities of the positive cell populations, the relative expression level of an antigen on CLL cells vs. normal cells could be determined. One antibody, scFv-2, consistently stained CLL cells less intensely than normal PBMC, whereas scFv-3 and scFv-6 both consistently stained CLL cells more brightly than normal PBMC. The fourth antibody, scFv-9, stained two of the five CLL

samples much more intensely than normal PBMC, but gave only moderately brighter staining for the other three CLL samples (Fig 8 and Table 2). This indicates that the antigens for scFv-3 and scFv-6 are overexpressed approximately 2-fold on most if not all CLL tumors, whereas scFv-9 is overexpressed 3 to 6-fold on a subset of CLL tumors.

CLL patients can be divided into two roughly equal groups: those with a poor prognosis (median survival time of 8 years) and those with a favorable prognosis (median survival time of 26 years). Several unfavorable prognostic indicators have been identified for CLL, most notably the presence of VH genes lacking somatic mutations and the presence of a high percentage of $CD38^+$ B cells. Since scFv-9 recognizes an antigen overexpressed in only a subset of CLL patients, we sought to determine if scFv-9 antigen overexpression correlated with the percentage of CD38+ cells in blood samples from ten CLL patients (Fig 11). The results indicate that scFv-9 antigen overexpression and percent $CD38^+$ cells are completely independent of one another.

Identification of antigens recognized by scFv antibodies by immunoprecipitation (IP) and mass spectrometry (MS)

To identify the antigens for these antibodies, scFvs were used to immunoprecipitate the antigens from lysates prepared from the microsomal fraction of cell-surface biotinylated CLL-AAT cells (Fig 12). The immunoprecipitated antigens were purified by SDS-PAGE and identified by matrix assisted laser desorption ionization mass spectrometry (MALDI-MS) or microcapillary reverse-phase HPLC nano-electrospray tandem mass spectrometry (μLC/MS/MS) (data not shown). ScFv-2 immunoprecipitated a 110 kd antigen from both RL and CLL-AAT cells (Fig12). This antigen was identified by MALDI-MS as the B cell-specific marker CD19. ScFv-3 and scFv-6 both immunoprecipitated a 45 kd antigen from CLL-AAT cells (not shown). This antigen was identified by MALDI-MS as CD23, which is a known marker for CLL and activated B cells. ScFv-9 immunoprecipitated a 50 kd antigen from CLL-AAT cells (Fig 12). This antigen was identified by μLC/MS/MS as OX-2/CD200, a known marker

for B cells, activated $CD4^+$ T cells, and thymocytes. OX-2/CD200 is also expressed on some non-lymphoid cells such as neurons and endothelial cells.

EXAMPLE 3

The capability of cells overexpressing OX-2/CD200 to shift the cytokine response from a Th1 response (IL-2, IFN-γ) to a Th2 response (IL-4, IL-10) was assessed in a mixed lymphocyte reaction using monocyte-derived macrophages/dendritic cells from one donor and blood-derived T cells from a different donor. As a source of OX-2/CD200-expressing cells, either OX-2/CD200 transfected EBNA cells as described below or CLL patient samples were used.

Transfection of 293-EBNA cells

293-EBNA cells (Invitrogen) were seeded at $2.5x10^6$ per 100mm dish. 24 hours later the cells were transiently transfected using Polyfect reagent (QIAGEN) according to the manufacturer's instructions. Cells were cotransfected with 7.2μg of OX-2/CD200 cDNA in vector pCEP4 (Invitrogen) and 0.8μg of pAdVAntage vector (Promega). As a negative control, cells were cotransfected with empty pCEP4 vector plus pAdVAntage. 48 hours after transfection, approximately 90% of the cells expressed OX-2/CD200 on their surface as determined by flow cytometry with the scFv-9 antibody.

Maturation of dendritic cells/macrophages from blood monocytes

Buffy coats were obtained from the San Diego Blood Bank and primary blood lymphocytes (PBL) were isolated using Ficoll. Cells were adhered for 1 hour in Eagles Minimal Essential Medium (EMEM) containing 2% human serum followed by vigorous washing with PBS. Cells were cultured for 5 days either in the presence of GM-CSF, IL-4 and IFN-γ or M-CSF with or without the addition of lipopolysaccharide (LPS) after 3 days. Matured cells were harvested and irradiated at 2000 RAD using a γ-irradiator (Shepherd Mark I Model 30 irradiator (Cs^{137})).

Mixed lymphocyte reaction

Mixed lymphocyte reactions were set up in 24 well plates using 500,000 dendritic cells/macrophages and 1 $x10^6$ responder cells. Responder cells were T cell enriched lymphocytes purified from peripheral blood using Ficoll. T cells were enriched by incubating the cells for 1 hour in tissue culture flasks and taking the non-adherent cell fraction. 500,000 OX-2/CD200 transfected EBNA cells or CLL cells were added to the macrophages/dendritic cells in the presence or absence of 30 μg/ml anti-CD200 antibody (scFv-9 converted to full IgG) 2-4 hours before the lymphocyte addition. Supernatants were collected after 48 and 68 hours and analyzed for the presence of cytokines.

Conversion of scFv-9 to full IgG

Light chain and heavy chain V genes of scFv-9 were amplified by overlap PCR with primers that connect the variable region of each gene with human lambda light chain constant region gene, and human IgG1 heavy chain constant region CH1 gene, respectively. Variable regions of light chain gene and heavy chain gene of scFv-9 were amplified with specific primers and the human lambda light chain constant region gene and the IgG1 heavy chain constant region CH1 gene were separately amplified with specific primers as follows:

R9VL-F1 QP: 5' GGC CTC TAG ACA GCC TGT GCT GAC TCA GTC GCC CTC 3' (SEQ ID NO 26);

R9VL/hCL2-rev: 5' CGA GGG GGC AGC CTT GGG CTG ACC TGT GAC GGT CAG CTG GGT C 3' (SEQ ID NO 27);

R9VL/hCL2-F: 5' GAC CCA GCT GAC CGT CAC AGG TCA GCC CAA GGC TGC CCC CTC G 3' (SEQ ID NO 28);

R9VH-F1: 5' TCT AAT CTC GAG CAG CAG CAG CTG ATG GAG TCC G 3' (SEQ ID NO 29);

R9VH/hCG-rev: 5' GAC CGA TGG GCC CTT GGT GGA GGC TGA GGA GAC GGT GAC CAG GGT GC 3' (SEQ ID NO 30);

R9VH/hCG-F: 5' GCA CCC TGG TCA CCG TCT CCT CAG CCT CCA CCA AGG GCC CAT CGG TC 3' (SEQ ID NO 31);

hCL2-rev : 5' CCA CTG TCA GAG CTC CCG GGT AGA AGT C 3'
(SEQ ID NO 32);

hCG-rev : 5' GTC ACC GGT TCG GGG AAG TAG TC 3'
(SEQ ID NO 33).

Amplified products were purified and overlap PCR was performed.

Final products were digested with Xba I/Sac I (light chain) and Xho I/Pin AI (heavy chain) and cloned into a human Fab expression vector, PAX243hGL. DNA clones were analyzed for PCR errors by DNA sequencing. The hCMV IE promoter gene was inserted at Not I/ Xho I site (in front of the heavy chain). The vector was digested with Xba I/Pin AI/EcoR I/Nhe I and a 3472 bp fragment containing the light chain plus the hCMV IE promoter and the heavy chain gene was transferred to an IgG1 expression vector at the Xba I/Pin AI site.

Cytokine analysis

The effect of the scFv-9 converted to full IgG on the cytokine profile in the mixed lymphocyte reaction was determined.

Cytokines such as IL-2, IFN-γ, IL-4, IL-10 and IL-6 found in the tissue culture supernatant were quantified using ELISA. Matched capture and detection antibody pairs for each cytokine were obtained from R+D Systems (Minneapolis, MN), and a standard curve for each cytokine was produced using recombinant human cytokine. Anti-cytokine capture antibody was coated on the plate in PBS at the optimum concentration. After overnight incubation, the plates were washed and blocked for 1 hour with PBS containing 1 % BSA and 5% sucrose. After 3 washes with PBS containing 0.05% Tween, supernatants were added at dilutions of two-fold or ten-fold in PBS containing 1%BSA. Captured cytokines were detected with the appropriate biotinylated anti-cytokine antibody followed by the addition of alkaline phosphatase conjugated streptavidin and SigmaS substrate. Color development was assessed with an ELISA plate reader (Molecular Devices).

As shown in Figure 14, the presence of OX-2/CD200 transfected but not untransfected cells resulted in down-regulation of Th1 cytokines such as IL-2 and IFN-

γ. Addition of the anti-CD200 antibody at 30 μg/ml fully restored the Th1 response, indicating that the antibody blocked interaction of OX-2/CD200 with its receptor.

As set forth in Figures 15 and 16, the presence of CLL cells in a mixed lymphocyte reaction resulted in down-regulation of the Th1 response. (Figure 15 shows the results for IL-2; Figure 16 shows the results for IFN-γ.) This was not only the case for cells over-expressing OX-2/CD200 (IB, EM, HS, BH), but also for CLL cells that did not over-express OX-2/CD200 (JR, JG and GB) (the expression levels for these cells are set forth in Figure 11). However, the anti-CD200 antibody only restored the Th1 response in cells over-expressing OX-2/CD200, indicating that for patients over-expressing OX-2/CD200, abrogating OX-2/CD200 interaction with its receptor on macrophages was sufficient to restore a Th1 response. In patients that did not over-express OX-2/CD200, other mechanisms appeared to be involved in down-regulating the Th1 response.

REFERENCES

The following references are incorporated herein by reference to more fully describe the state of the art to which the present invention pertains. Any inconsistency between these publications below or those incorporated by reference above and the present disclosure shall be resolved in favor of the present disclosure.

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It will be understood that various modifications may be made to the embodiments disclosed herein. For example, as those skilled in the art will appreciate, the specific sequences described herein can be altered slightly without necessarily adversely affecting the functionality of the polypeptide, antibody or antibody fragment used in binding OX-2/CD200. For instance, substitutions of single or multiple amino acids in the antibody sequence can frequently be made without destroying the functionality of the antibody or fragment. Thus, it should be understood that polypeptides or antibodies having a degree of homology greater than 70% to the specific antibodies described herein are within the scope of this disclosure. In particularly useful embodiments, antibodies having a homology greater than about 80% to the specific antibodies described herein are contemplated. In other useful embodiments, antibodies having a homology greater than about 90% to the specific antibodies described herein are contemplated. Therefore, the above description should not be construed as limiting, but merely as exemplifications of preferred embodiments. Those skilled in the art will envision other modifications within the scope and spirit of this disclosure.